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Body-wall muscle restoration dynamics are different in dorsal and ventral blastemas during planarian anterior regeneration

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ABSTRACT. Planarians are simple, acoelomate, triploblastic organisms with a remarkable capacity of regeneration. In the last years, several specific cellular and molecular markers have been used to study this biological problem in these organisms. Here, we monitor body-wall musculature restoration during anterior regeneration through confocal microscopy and using a monoclonal antibody called TMUS-13, which recognizes the myosin heavy-chain of muscle cells. We have found differences in the dynamics of muscle pattern restoration between dorsal and ventral surfaces of the growing blastemas, especially during the first days of regeneration. Blastema contains old longitudinal fibers coming from the postblastema throughout all the regenerative process. These fibers could have a role in supporting the growing blastema and/or guiding the entry of different cell types from the postblastema region. New longitudinal fibers within the blastema seem to appear from outgrowing processes of the existing longitudinal fibers. On the other hand, new circular fibers appear *de novo* within the regenerative blastema. Finally, the original muscle pattern seems to be restored through intercalation of new muscle fibers throughout an initial muscle scaffold.

KEY WORDS: Platyhelminthes, planarian, body-wall muscle, regeneration, blastema.

INTRODUCTION

Pattern restoration during planarian regeneration remains, at present, obscure, mainly because of the lack of clear molecular approaches to solving this biological question (for a general review, see BAGUÑÀ (1998). In the last few years, however, several molecular studies, some using cell-type specific molecular markers, have been carried out (BUENO et al., 1997; BAYASCAS et al., 1998; SÁNCHEZ & NEWMARK, 1998; AGATA & WATANABE, 1999; KATO et al., 1999; KOBAYASHI et al., 1999). Recently, we started studying myocyte differentiation and body-wall muscle pattern restoration during planarian regeneration through immunostaining with a monoclonal antibody specific to planarian muscle cells. This antibody, called TMUS-13 (ROMERO et al., 1991; BUENO, 1994), recognizes the myosin heavy-chain protein from both mature muscle cells and differentiating myocytes (CEBRIÀ et al. 1997). Planarians have a well-developed body-wall musculature, which gives rise to a complex muscle network throughout the organism. This muscle network somehow makes up for the lack of a true skeletal system in these animals and supports all other kinds of cells. During regeneration muscle fibers perform an early function by closing the wound through a strong contraction of the body-wall (CHANDEBOIS, 1980). It is not clear, however, if these muscle fibers have a role in blastema formation and growth. Here, we describe how this muscle pattern is restored during anterior regeneration. In contrast with the results shown in a previous paper where we did not check for differences between dorsal and ventral blastemas (CEBRIÀ et al., 1997), we found differences in the dynamics of muscle restitution between dorsal and ventral surfaces of the blastema. These differences reflect the different patterns already observed in intact planarians and suggest that, at least ventrally, the blastema seems to contain, during the entire regenerative process, old muscle fibers coming from the postblastema (a narrow strip of old tissue close to the wound and where high proliferative activity is detected from the first hours of regeneration,

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Saló & Baguñà, 1984). The possible role of these existing muscle fibers as a support of the growing blastema and/or as a guide for the entry of myocytes or other cell types from the postblastema is discussed. Finally, we also discuss whether muscle pattern restoration is carried out through a distoproximal, proximodistal or intercalary sequence of events.

MATERIAL AND METHODS

Species and culture conditions

The planarians used belong to a diploid (2n=8) and asexual strain of the species *Schmidtea mediterranea* (Baguñà, 1973, Benazzi et al., 1975) collected in a small fountain in Barcelona (Catalunya, Spain). For anterior regeneration experiments, organisms 5-8 mm long were cut at a post-cephalic level and kept at 17±1ºC. All the organisms were starved for 15 days before use.

Whole-mount immunostaining with TMUS-13

Intact and regenerating organisms were treated with 2% HCl in distilled water for 30 s, which kills planarians instantly and leaves them completely flat. Then, they were fixed in Carnoy's solution (EtOH, chloroform and glacial acetic acid in proportions 6:3:1) for 2 hr at room temperature (RT). After fixation animals were washed in 75% MeOH in PBS for 5 min, bleached in 6% hydrogen peroxide (H_2O_2) in MeOH for 4-6 hr under light, and washed in MeOH 3 x 5 min. The animals were then rehydrated in a decreasing series of MeOH in PBST (PBS-0.3% Triton X-100), 5 min in each step. After a 5 min wash in PBST, animals were blocked in 0.25% bovine serum albumin (BSA) in PBST for 2 hr at RT and then incubated with the anti-myosin heavy chain monoclonal antibody TMUS-13 (Romero et al., 1991; Bueno, 1994, Cebrià et al., 1997) diluted 1/10 in 0.25% BSA in PBST for 16-24 hr at RT (shaking). The samples were then washed in 0.25% BSA in PBST for 6-12 hr (several changes of the medium) and TMUS-13 was detected with secondary goat-anti-mouse conjugated to fluorescein (GAM-FITC; Sigma) diluted 1:75 in 0.25% BSA in PBST for 16-20 hr in the dark. After washing in PBST for several hours and with several changes of the medium, specimens were mounted in Vectashield medium (Vector Laboratories, Inc.) and their fluorescence was detected with confocal microscopy.

Confocal microscopy

Confocal laser scanning microscopy (CLSM) was performed with a Leica TCS 4D (Leica Lasertechnik, Heidelberg) adapted to an inverted microscope (Leitz DMIRB). Images were taken using a x40 (NA 1.0) Leitz Plan Fluotar lens. Pictures shown correspond to 3D reconstruction from several collected images, each of them being the average of eight line scans at the standard scan rate. All the images are in the same pseudo-color (glow look-up table).

RESULTS

Cephalic body-wall muscle pattern is different in dorsal and ventral surfaces

As has been previously described, the body-wall muscle pattern of the most anterior region of planarians is different between dorsal and ventral surfaces (Cebrià et al., 1997). These differences are more evident for longitudinal muscle fibers. Dorsally, these longitudinal muscle fibers seem to converge upon a central zone close to the most anterior tip of the organism (Fig. 1A; arrows). In contrast, on the ventral surface the longitudinal fibers run in parallel as they reach the anterior border (Fig. 1B; arrows).

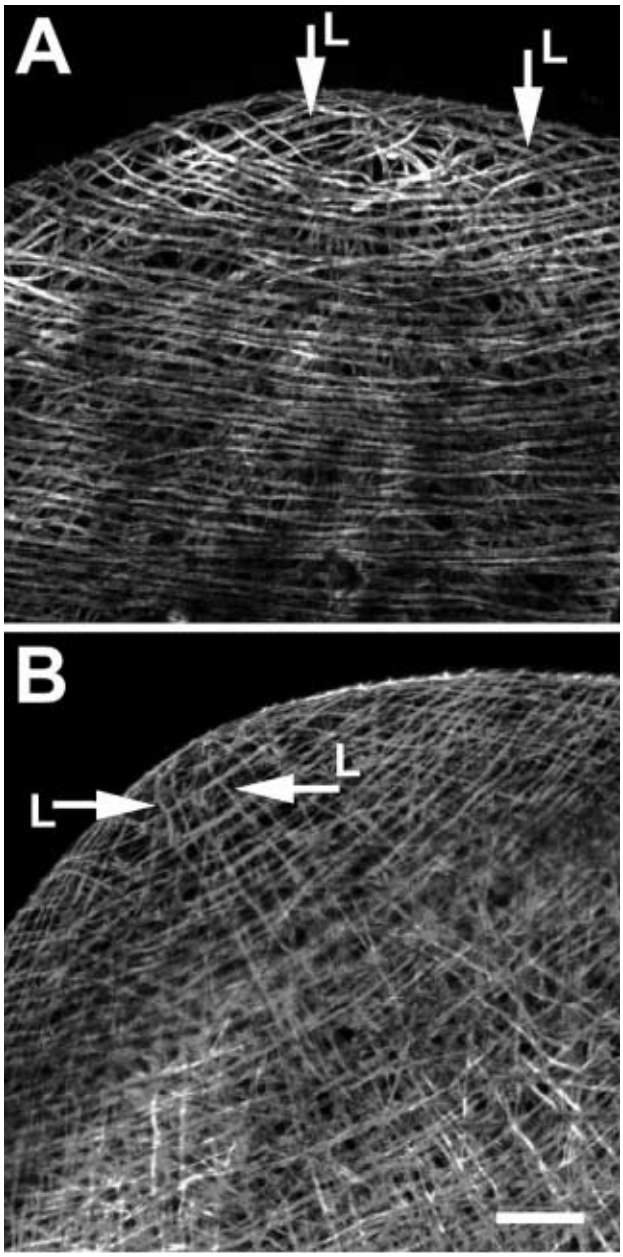

Fig. 1. – 3D projection from confocal microscopic images of body-wall musculature of the cephalic region immunostained with TMUS-13. (A) Dorsal view, with longitudinal fibers (arrows) converging to a central region, and (B) Ventral view, with longitudinal fibers (arrows) running in parallel. *L*, longitudinal fibers. *Scale bar* 50 μm.

Body-wall muscle pattern restoration during anterior regeneration

For anterior regeneration experiments animals were amputated at a post-cephalic level and the appearance of the new muscle pattern in the forming head was monitored with TMUS-13 and confocal microscopy. Fig. 2 shows how this muscle pattern is restored within the first 6 days of regeneration. At day 1 of regeneration the blastema is still rather small and difficult to distinguish. Dorsally, in the most anterior region and where the blastema is being formed, there appears a big "hole" lacking muscle fibers and delimited by rather disorganized old muscle fibers (Fig. 2A). This "hole" is neither a fixation artefact nor a blastema breaking, as it is really filled with many cells that are different from muscle cells. In contrast, the muscle fibers of the ventral surface keep a more organized pattern similar to that found in an intact organism (Fig. 2B). At day 2 of regeneration there is a significant increase in muscle fibers and differentiating myocytes within the dorsal "hole", although the pattern is still rather disorganized. It can be seen how existing longitudinal fibers enter the blastema (Fig. 2C; arrows). However, the blastema ventral musculature continues to be formed, apparently, by old existing fibers with a more organized pattern. This organization is seen more clearly in the longitudinal fibers, whereas circular fibers are absent within the blastema. In the most distal part of this blastema, the muscle pattern is partially lost, even when compared with a one-day regenerant. Distally, these ventral muscle fibers are arranged in a more relaxed pattern with wider distances between them (Fig. 2D). At day 3 of regeneration an incipient arrangement of the muscle fibers is seen on the dorsal surface of the blastema. These fibers

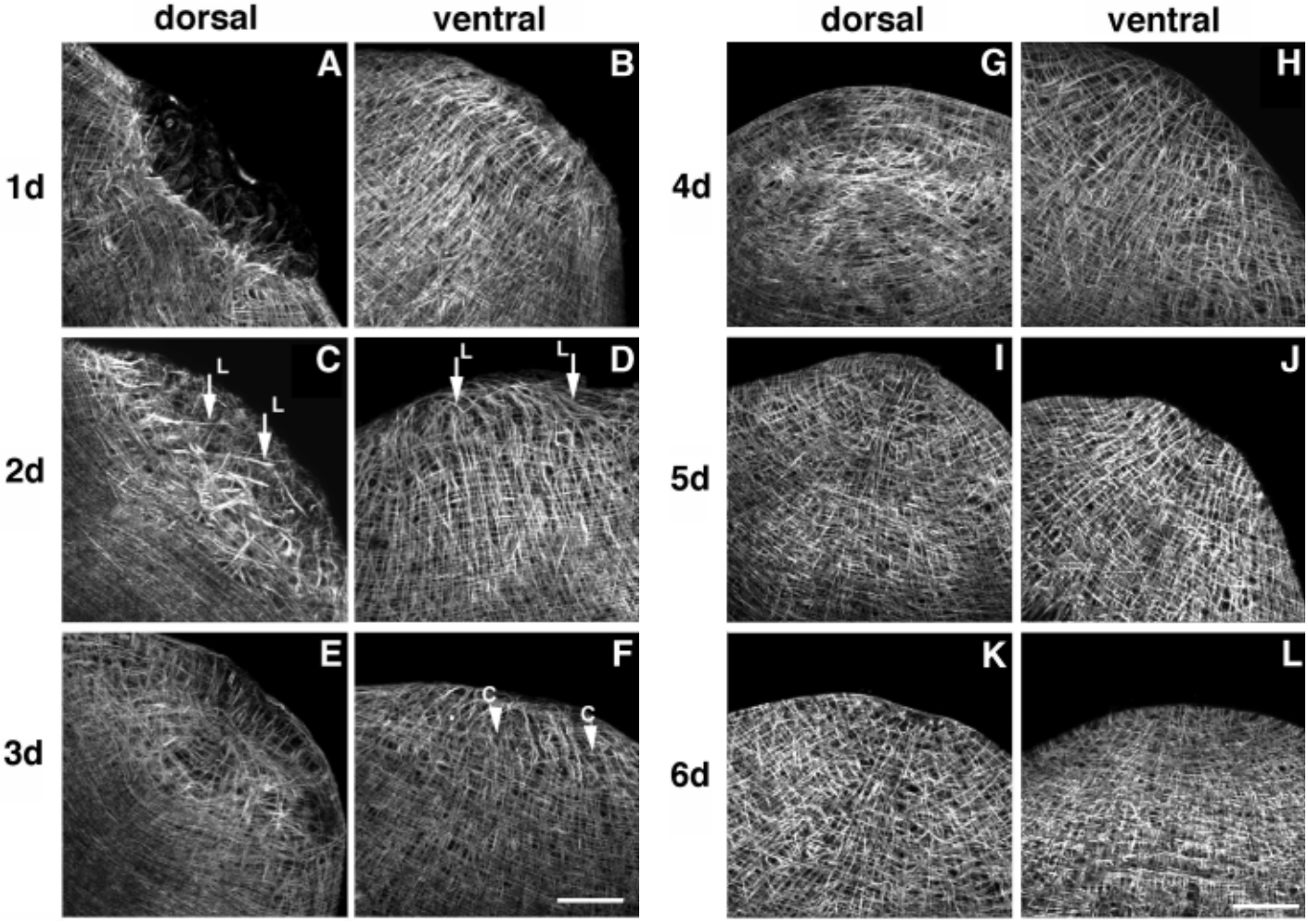

Fig. 2. – 3D projection from confocal microscopic images of regenerating heads immunostained with TMUS-13. Day 1 regenerant, dorsal (A) and ventral (B) views. Note a dorsal "hole" lacking muscle fibers. Day 2 regenerant, dorsal (C) and ventral (D) views. Existing dorsal longitudinal muscle fibers enter the blastema (arrows). Ventrally, the blastema only contains existing longitudinal fibers (arrows). Day 3 regenerant, dorsal (E) and ventral (F) views. Muscle fibers on the dorsal surface organize in a first outline of the definitive pattern, converging to a central region of the blastema. On the ventral surface of the blastema, new circular fibers appear (arrowheads). Day 4 regenerant, dorsal (G) and ventral (H) views. The muscle pattern in both dorsal and ventral surfaces is already restored. In the following days a more dense and compact muscle pattern appears (I-L). From this moment, cephalic muscle pattern can be considered restored. *L*, longitudinal fibers, *C*, circular fibers. Scale bar: 50 μm.

tend to converge to a central region of the blastema in a similar way to what occurs for an intact head (Fig. 2E). In the ventral surface, and in the distal part of the blastema, new thin circular fibers are formed (Fig. 2F; arrowheads). From the fourth day of regeneration the muscle pattern, both dorsal and ventral, is restored and in the following days the number of fibers increases, resulting in a more compact and dense pattern similar to the one found in intact organisms (Fig. 2G-L).

DISCUSSION

Biological significance of the differences described in dorsal and ventral blastemas

The differences observed between dorsal and ventral surfaces, mainly during the first days of regeneration, seem to reflect the differences found in an intact head which could be just indicating, at a structural level, how this muscle network is closed in the most anterior tip. We cannot exclude, however, that these differences are related to the way the epithelium heals the wound. One of the unsolved questions about planarian regeneration is how the regenerant fragments "know" which parts (anterior or posterior) are lacking and, consequently, have to be regenerated. CHANDEBOIS (1976; 1980) suggested that during anterior regeneration it is the dorsal epithelium which expands to close the wound. In contrast, the ventral epithelial cells would heal the wound in posterior regeneration. These different ways of wound closure would give the specific signals for anterior or posterior regeneration. If CHANDEBOIS' hypothesis is right, this dorsal region lacking muscle fibers could be related in some way to the expansion of the dorsal epithelial cells to close the wound. To corroborate this hypothesis, we should study how the muscle fibers are arranged at the caudal end of the organism as well as what happens during muscle pattern restoration in posterior regenerants. In fact, SALVENMOSER et al. (this volume) show how during posterior regeneration in the microturbellarian *Macrostomum* sp., the blastema is shifted ventrally and the differences between dorsal and ventral muscle patterns reverse the ones observed during planarian anterior regeneration.

Planarian regenerative blastema contains old muscle fibers from the postblastema

What we can say at present is that, from the beginning, the blastema seems to contain, at least ventrally, existing muscle fibers coming from the postblastema. These muscle fibers, mainly longitudinal, are detected within the blastema during the entire regenerative process, which suggests that they could play a role in supporting the growing blastema and/or in guiding the entry of myocytes from the postblastema. At day 1 of regeneration many myocytes appear in the postblastema, mostly on the ventral surface. As regeneration proceeds, they migrate into the blastema where they differentiate into new muscle fibers (unpubl. data). Therefore, the appearance of these myocytes close to the existing longitudinal muscle fibers suggests that the myocytes could use these fibers in their migration into the blastema. This association between myocytes and mature muscle fibers has also been described within the planarian pharynx (CEBRIÀ et al., 1999). Moreover, REITER et al. (1996) have shown how, during embryonic development of some platyhelminths, existing muscle fibers can guide the appearance of the new fibers.

Muscle pattern restoration: distoproximal, proximodistal or intercalary?

When considering muscle pattern restoration, one of the questions to answer is whether this restitution is carried out in a distoproximal or proximodistal sense; in this case, proximal is the region closest to the wound or postblastema, and distal is the tip of the growing blastema. We have to distinguish this question, which refers to the differentiation of the muscle pattern, from the distoproximal sequence of events through which the different regions (i.e. head, prepharyngeal or pharyngeal regions) are determined during regeneration (WOLFF et al., 1964; SALÓ, 1984). The results shown in Fig. 2 do not let us give a definitive answer to this question. At day 2 of regeneration the blastema contains many muscle fibers, but these are rather disorganized. At day 3, however, an incipient pattern can be seen dorsally, with new fibers converging to a central region of the blastema, as happens in an intact head. So, it seems that in few hours the fibers within the blastema organize themselves rapidly into a first outline of the definitive pattern, which will be completely restored from this initial muscle scaffold. Whereas new longitudinal muscle fibers within the blastema (especially on the dorsal surface) seem to appear from the outgrowth of existing longitudinal fibers coming from the postblastema, the circular fibers probably appear *de novo* within this blastema, as is suggested by comparison of dorsal and ventral blastemas between day 2 and day 3 of regeneration. At present we do not know how new circular fibers become oriented within the blastema, though it is possible that longitudinal fibers can be involved in this process. SALVENMOSER et al. (this volume) show how, during body-wall muscle regeneration in *Macrostomum* sp., the future circular fibers appear as myocytes perpendicularly oriented to the longitudinal fibers. The final restoration of the muscle pattern could then occur in proximodistal sequence, as is suggested more clearly for the appearance of the new muscle fibers within the blastema, or through an intercalary differentiation of the new fibers throughout all this muscle scaffold detected at day 3. A similar intercalary mechanism has been proposed for muscle pattern restoration during pharynx regeneration (ESPINOSA, unpubl.; BUENO et al., 1997) and cell-renewal (CEBRIÀ et al., 1999).

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